

Salty Microbiology

*A safe, low-cost
classroom activity
focuses on
hypersaline
microbial ecology*

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Using microbiology activities in the classroom is an effective way for teachers to address National Standards in the life sciences. However, common microbiology activities that involve swabbing doorknobs and hands are too risky due to the likelihood of culturing human pathogens. In addition, making sterile media and maintaining sterile conditions can be difficult, and activities requiring aseptic techniques are particularly challenging for the lower grades.

To address these problems, we have developed a microbiology system that focuses on a specialized class of microbes that can live under extreme hypersaline conditions. The high salt content of the media (20 percent or more) eliminates the need for sterilization and aseptic techniques, and rules out the possibility of culturing bacterial pathogens. Furthermore, the activity has been developed with the use of low-cost, common household materials.

Microbes from extreme environments are models of evolutionary selection and specialization. Such microbes may have played a role in the origin of life on Earth (in the concentrated brine of tidal pools often envisioned), and are a key focus in the search for extraterrestrial life in hypersaline environments that may exist on Mars and Europa.

Media components and tools

Preparing microbiological media requires an understanding of nutrition and physiology. Most standard microbiology media are termed *complex media* because they contain components that have not been chemically defined. Yeast extract, tryptone, and casein, for example, are commonly used as carbon, nitrogen, and energy sources to provide a rich source of nutrients for a wide variety of microbes.

Halotolerant microbes (organisms that adapt to conditions of high salinity) are typically grown on rich complex media that include a mixture of salts. However, students can design individual growth media by substituting these laboratory reagents with materials obtained from supermarkets. Students may try simple table sugar as a carbon and energy source, for example, or complex ingredients such as nonfat dry milk, oatmeal, cornmeal, dry yeast, molasses, corn syrup, or infant formula (which also includes protein as a nitrogen source). Mineral nutrients, including nitrogen, can be derived from plant food, and vitamin pill extracts can provide trace minerals.

The key to creating a hypersaline microbiology system is the addition of high concentrations of salt [a mixture of table salt (NaCl) and a salt substitute (KCl)] to the growth media. No extremely halotolerant bacterial or archaeal pathogens are known. *Staphylococcus* is relatively halotolerant, but it does not grow above 15 percent (w/v) salinity. High salt concentrations of 25 percent or more, and the addition of magnesium (as magnesium sulfate in Epsom salts to 1 percent), will often enrich for haloarchaea — brightly colored red or pink organisms that depend on high salt concentrations for growth. Therefore, salt concentrations of 20 percent to 25 percent (w/v) should be used in this activity.

Although pathogenic bacteria will not grow at high salt concentrations, some fungi can grow under these desiccating conditions. Fungi are unlikely to be pathogenic, but can present problems for immunocompromised individuals. Fungal growth can be eliminated in the plates by adding a relatively inexpensive antifungal agent such as nystatin or by using over-the-counter antifungal preparations.

Solid media plates allow for the isolation and examination of individual microbial species. Students can observe the colonies and choose which ones to

investigate. Microbiological media can be solidified using gelatin, although this is less reliable and often more costly than agar. Research laboratories use agar that is highly purified and tested, costing \$100 or more per pound. Fortunately, less expensive substitutes are readily available. Internet vendors distribute food-quality agar for use as a gelatin. Often called agar-agar, this material is inexpensive, less than \$20 per pound, and performs as well as the research-grade product. The agar is used at a rate of 15 gL⁻¹, with each liter providing two, 20-plate sleeves of standard 100 mm Petri dishes. Each student or group should be able to finish the exercise with one sleeve of plates. Agar-agar works well for gel electrophoresis, as well.

Plastic or glass Petri dishes are available, with the latter being reusable. However, any flat plastic container can be a suitable mold for solid media. Plastic food storage containers work well since the media and dishes in this activity do not have to be exposed to sterilizing heat. The solid media can be used to pour slants in test tubes for long-term maintenance of microbial isolates. Cultures of hypersaline microbes on plates or slants should be kept in a moist chamber to prevent drying of the surface. A plastic storage box with a lid (does not have to seal tightly) works well when a cup of water is placed inside with the cultures.

Standard aseptic technique uses a flame to sterilize metal bacterial loops. The hypersaline system does not require aseptic technique. Bacterial loops can be made from thin bare wire attached to a wooden handle, but perhaps the easiest way to transfer colonies from plate to plate for isolation and maintenance is simply to use wooden toothpicks. Liquid cultures can be kept in any bottle that has a loose-fitting top. Daily swirling of the culture provides aeration. Transfers can be made with eyedroppers or with bacterial loops. Before disposal or washing, any materials contaminated with microbes should be treated with household bleach for one hour or more.

Making the media

Students can choose what materials to add to their media, however certain combinations have a greater chance of success. Figure 1 (p. 42) provides a recipe for one medium that works well. Some hints are given below to help in media preparation.

Individual carbon sources such as glucose or glycerol (glycerin) can be included at 1 percent to 5 percent of the mixture in place of the milk solution as a carbon and energy source. Oatmeal, baby formula, and cornmeal can be used instead of the milk as well. All-purpose plant food has worked well as a source of nitrogen and phosphorus and should be included along with the vitamin extract (no matter which car-

bon and energy sources are chosen). A vitamin pill that has as many trace minerals as possible—such as manganese (Mn), molybdenum (Mo), and cobalt (Co)—should be used. The vitamin pill and molasses provide complex nutrients required by more fastidious organisms. The dissolved pill suspension can be filtered using a funnel and laboratory filter paper or using a coffee filter. As stated, salt must be added at 20 percent or more to provide the selection pressure needed to suppress the growth of unwanted microbes.

A common athlete's foot treatment containing 1 percent clotrimazole as a topical liquid was easy to work with and effective when added directly to the media. It also can be topically applied to solid media plates, but is not as effective. Other fungicides, including a common topical fungicide containing 1 percent tolnaftate, a broad-spectrum liquid fungicide (12.5 percent chlorothalonil), and a fungicide powder (80 percent maneb powder), were not effective. Nystatin can be incorporated directly into the medium at 10,000 UL⁻¹ after cooling to about 45°C, and although this is a laboratory chemical, it is a less expensive alternative to the 1 percent clotrimazole solution.

FIGURE 1

Medium recipe of approximately 250 mL (1 cup).

For this description, the amounts are given in metric units, with approximate kitchen measurements in parentheses.

Mix and boil the following ingredients until the agar or gelatin dissolves:

- ◆ 50 g (5 tsp) iodine-free table salt (NaCl)
- ◆ 1.25 g (1/8 tsp) salt substitute
- ◆ 0.5 g (1/8 tsp) all-purpose plant food
- ◆ 5 g (3/4 tsp) unsulphured molasses
- ◆ 3.75 g (1.5 tsp) agar-agar or 33.6 g (12 tsp) gelatin
- ◆ 180 mL (3/4 cup) tap water

After cooling the mixture to about 45°C (baby-bottle temperature), add the following:

- ◆ 50 mL (10 tsp) of milk solution—2.5 g (1.5 tsp) instant nonfat dry milk in 50 mL (10 tsp) tap water
- ◆ 20 mL (4 tsp) vitamin solution—dissolve 1 multivitamin tablet in 50 mL (10 tsp) tap water with low heat (< 45°C) and decant or filter
- ◆ 1 mL (5 drops) antifungal preparation (1 percent clotrimazole solution)

Source of halotolerant microbes

There are many hypersaline areas throughout the United States and the world, including salt lakes, soda lakes, sabkhas, playas, salt flats, solar salterns, and salt licks. The Salt Plains Microbial Observatory collects halotolerant microbes from the Great Salt Plains of Oklahoma. Halotolerant microbes also can be obtained from a variety of widely available prepared foods. However, many foods that might be considered high in salt are either not salty enough to harbor microbes that grow at 20 percent salinity or have salt added after fermentation. Soy sauce, for instance, is generally at 15 to 20 percent salinity, but much of this salt is added after fermentation, and no highly halotolerant organisms are present. Readily available commercial sources of halotolerant organisms include a variety of salted fermented fish sauces available from Asian markets or Internet vendors. Fish pastes and sauces include jeotgal and chotkal from Korea, bagoong from the Philippines, and mắm nêm from Thailand. Five brands of fish sauce produced in Thailand and Viet Nam were tested and all yielded halotolerant microbial isolates at 20 percent salinity. Different microbes were observed at room temperature (25°C) and 37°C. Growth rates of the isolates varied, but were generally slow with colonies often taking several days to develop.

Teachers have some options for introducing halotolerant microbes in the classroom. In some instances, teachers may allow students to try isolating halotolerant microbes from different sources for study (see sidebar “Fishing for salty microbes,” p. 42). However, most curricula will not have room for this research-oriented activity that invariably takes more time and has a greater risk of failure. Therefore, the teacher can choose to provide microbes that they isolated earlier, purchased from culture collections, or obtained gratis from researchers in the field.

The pre-isolated microbes can be provided to students as pure cultures on solid media plates or in liquid media (such as the ones described above without agar and gelatin), but also can be incorporated into mock soils. Sandy soil (or simply sand) can be soaked in a saturated salt solution and dried. Then, the “hypersaline soil” can be moistened with dense liquid microbial culture (a few drops of culture per gram). A pea-sized crumb of this mixture spread on the surface of a standard 100 mm agar plate is enough to plate the soil. Without using a lot of in-class time, this introduction allows students to “isolate” microbes from the soil, and have a high likelihood of success. When tested in the classroom, two microbes of different colors (red and pink) were added to the hypersaline soils. Students could easily distinguish the two isolates and direct their isolation efforts accordingly.

Microbial ecology context

The protocols reported here become more powerful when put in the context of microbial ecology and physiology. The role that nutritional needs and environmental conditions play in shaping an ecosystem and driving evolution can be incorporated into lessons surrounding this classroom activity. Students can form hypotheses about the requirements of living systems and then test those hypotheses by examining the microbial isolates. The lessons can include material on biogeochemical cycles and the roles that microbes play in global ecology. Characterization of the isolates through biochemical and physiological tests can bring in aspects of subcellular components and metabolic machinery.

The study of life in extreme environments acts as a counterpoint to commonplace mesophilic organisms. The diversity of microbes and their metabolic capabilities is far greater than that of the plants and animals currently emphasized. Microbes can eat rock, breathe rock, and thrive under extremely inhospitable conditions. Microbes that live in hypersaline systems and develop mechanisms to overcome extreme osmotic conditions can be used as examples of natural selection and specialization.

In addition, hypersaline systems on Earth have relevance to astrobiology, which is always fascinating for students. Mars apparently has salty soils resulting perhaps from salty seas, while Jupiter's moon Europa may have a salty sea below its icy crust. Many of the microbes found in hypersaline environments are Archaea, although bacteria and fungi are highly abundant as well. Discussions of Archaea can be used to transition students from obsolete, but accessible, models of the tree of life, such as the Whitaker five-kingdom tree based on morphology, to the more widely accepted three-domain tree based on phylogenetic analyses of rRNA gene sequences (see sidebar "Full-circle molecular microbial ecology"). ■

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Acknowledgment

This work has been supported by grants from the National Science Foundation Microbial Observatories program (MCB-0131659), the Kansas Honor Regents Academy, and the Watkins Foundation.

Fishing for salty microbes.

Hypersaline environments are found around the world. Samples from any of these could be used as inocula for the isolation of halotolerant microbes. Advanced classes may be able to take advantage of this type of research-oriented activity, collecting soils or waters from nearby salt licks or salt lakes. Salted foods and household products can be examined as sources of halotolerant microbes. Although halotolerant organisms may be present in the following salty products, no isolates were obtained from the initial screening (at 20 percent salinity) of beef jerky, dishwashing liquid, beef bouillon granules, laundry detergent, or soy sauce. Other pure salt products such as sea salt, thawing salt, and table salt did not yield halotolerant microbes. Several of these products were tested at 10 or 15 percent salinity, and a variety of microbes were obtained, some of which may have been able to grow at 20 percent salinity. Advanced students can work towards isolating bacteria from these and other sources, later characterizing their salt tolerances.

Full-circle molecular microbial ecology.

Research in microbial ecology has greatly benefited from advances in DNA technologies. Scientists can now use gene sequences to mathematically compare organisms, generating trees of relatedness that have phylogenetic and evolutionary relevance. The gold standard has become rRNA gene sequences, which are found in all cellular organisms. These are highly conserved genes, but with sufficient variability for good resolution of taxonomic differences. In fact, rRNA gene sequences can be obtained from direct extracts of genomic DNA from soils. Given that only 1 percent or less of environmental microbes can currently be grown in the laboratory, these culture-independent molecular techniques provide a more accurate picture of the microbial community. The Salt Plains Microbial Observatory has been isolating and characterizing organisms from hypersaline terrestrial systems. DNA is extracted from each isolate and PCR is used to amplify rRNA genes. These genetic sequences are used to generate trees of relatedness, phylogenetic trees, which place the unknown isolate in the context of all of the organisms reported to GenBank. This activity draws clear connections between the chemical basis of heredity and the genetic basis of speciation. The hypersaline microbiology system has been coupled with classroom activities that include DNA extraction by freeze-thaw cycles, faux PCR reactions, and online analysis of sequences provided to students as files after faux sequencing of the PCR amplicons. In a research setting, the gene sequences would be added to GenBank, completing a process that has become known as full-circle molecular microbial ecology.